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(*S*)- and (*R*)-Eriodictyol-6-*C*-*β*-D-glucopyranoside, novel keys to the fermentation of rooibos (*Aspalathus linearis*)

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Abstract

The processed leaves and stems of *Aspalathus linearis* contain a new diastereomeric pair of the flavanones, (*S*)- and (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside, which is also formed via the oxidative cyclization of the dihydrochalcone, aspalathin, under conditions which mimic the fermentation process. © 2000 Published by Elsevier Science Ltd.

Keywords: *Aspalathus linearis*; Leguminosae; Rooibos tea; Phenolic metabolites; Flavanone glucoside; Fermentation; Dihydrochalcone

1. Introduction

Rooibos (*Aspalathus linearis*) is a unique South African shrub, cultivated for the production of the increasingly popular beverage, rooibos tea. Rooibos tea has been used by indigenous people, the Khoikhoi, since 1772 (Morton, 1982) and the tea itself, as well as a variety of compounds isolated from it, are claimed to have a number of physiological properties, including antioxidant activity (Joubert and Ferreira, 1996).

Rooibos leaves, similar to those of *Camellia sinensis*, are processed to develop the characteristic organoleptic qualities of rooibos tea. This "fermentation process" includes shredding and bruising of the plant material, followed by exposure of the moistened material to sunlight at 30–35°C. According to Joubert and Ferreira (1996) the content of the dihydrochalcones, aspalathin (**1**) and nothofagin (**2**), the major ethyl acetate soluble polyphenols in unprocessed rooibos, decreases significantly with processing. We thus mimicked the "fermentation process" of rooibos with aspalathin (**1**) as substrate under various conditions and also continued the investigation of the phenolic compounds of this commercially viable plant aimed at establishing a reliable chemical profile. Here, we report the isolation and structural elucidation of a new pair of flavanone C-glucosides, which was also synthesized via biomimetic methodology.

2. Results and discussion

2.1. Structure elucidation of (*S*)- and (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside (**4**)

Purification of the acetone extract of the commercial form of *A. linearis* with size-exclusion chromatography (Sephadex LH-20) and adsorption chromatography (Cellulose MN 300) (Rabe et al., 1994) afforded a mixture of (*S*)- and (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside (**4a** and **4b**) as a yellow amorphous solid. The free

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Table 1
^{1}H NMR data of diastereomeric mixture **4** at 300 MHz and 296 K[a]

Ring	H	Acetone-d_6	Ethanol-d_6
A	8	5.92 (*br.s*)	6.02 (*s*)
	5-OH	12.70 (*br.s*)	–
B	2	7.15 (*br.d*, 3.3)	6.93 (*d*, 2.0)
	5	6.87–6.83 (*m*)	6.80 (*d*, 8.4)
	6	6.87–6.83 (*m*)	6.74 (*dd*, 8.4, 2.0)
C	2	5.39, 5.38[b] (*dd*, 12.0, 3.0)	5.26 (*dd*, 12.5, 3.5)
	3_{cis}	2.75, 2.72[b] (*dd*, 17.2, 3.0)	2.73 (*dd*, 17.0, 3.5)
	3_{trans}	3.14, 3.13[b] (*dd*, 17.2, 12.0)	3.03, 3.02[b] (*dd*, 17.0, 12.5)
Glc	1	4.85 (*d*, 9.6)	4.79 (*d*, 10.0)
	2	3.72 (*dd*, 9.6, 8.5)	4.11 (*br.dd*, 10.0, 10.0)
	3	3.60 (*dd*, 9.5, 8.5)	3.50–3.45 (*m*)
	4	3.51 (*dd*, 9.5, 8.0)	3.50–3.45 (*m*)
	5	3.49–3.41 (*m*)	3.41–3.34 (*m*)
	6	3.84-3.78 (*m*)	3.84 (*br.dd*, 12.8, 2.9), 3.71[b] (*br.dd*, 12.6, 4.9)

[a] Splitting patterns and *J*-values (Hz) are given in parentheses.
[b] Duplicated resonances.

phenolic forms **4a** and **4b** and their peracetates were inseparable[3] due to the high polarity of these compounds. NMR, MS and UV studies were thus performed on the diastereomeric mixture, **4**.

Diastereomeric eriodictyol-6-*C*-*β*-D-glucopyranoside (**4**) showed an [M$^+$] at *m*/*z* 450.1161 (calcd. 450.1162) corresponding to the molecular formula $C_{21}H_{22}O_{11}$. The presence of the flavanone skeleton was evident from the UV (λ_{max} 289 nm, 335 nm), ^{1}H NMR (δ 5.39 and 5.38, *dd*, $J = 3.0$ and 12.0 Hz for H-2; 2.75 and 2.72, *dd*, $J = 3.0$ and 17.2 Hz for H-3_{cis}[4]; 3.14 and 3.13, *dd*, $J = 12.0$ and 17.2 for H-3_{trans}, acetone-d_6) and ^{13}C NMR (δ_C 43.1536 and 42.9208 for C-3; 197.052 for C-4) spectra. The ^{1}H NMR spectrum in acetone-d_6 indicated the presence of a chelated hydroxyl group (δ 12.70), an A-ring singlet as well as the characteristic seven-spin system of the protons of a *β*-D-glucopyranosyl moiety C–C coupled at C-1″ to the aglycone ($^3J_{1\Delta}$, $2\Delta = 9.6$ Hz) (Koeppen and Roux, 1965) (Table 1). Individual sugar protons were identified by means of a ^{1}H–^{1}H COSY experiment. The substitution pattern of the B-ring was elucidated from the ^{1}H NMR spectrum in ethanol-d_6, showing a distinct ABX system (δ 6.93, *d*, $J = 2.0$ Hz, H-2′; δ 6.80, *d*, $J = 8.4$ Hz, H-5′ and δ 6.74, *dd*, $J = 8.4$, 2.0 Hz, H-6′) which confirmed the presence of a catechol ring. From the FAB mass spectrum, fragments characteristic of Retro–Diels–Alder fragmentation with loss of CO provided additional evidence of a catechol B-ring (*m*/*z* 136) as well as a phloroglucinol A-ring with a C–C linked glucopyranosyl substituent (*m*/*z* 288). Carbon resonances (Table 2) were allocated by means of HETCOR and HMBC experiments. In an HMBC experiment (Fig. 1, acetone-d_6) correlations of H-1″ (δ 4.85) of the glucopyranosyl substituent and the chelated hydroxyl group (δ 12.70) to the same carbon (δ_C 105.283) proved the latter to be C-5, and thus, that the *β*-D-glucopyranosyl substituent is linked to C-6 of the eriodictyol aglycone. This regiochemistry is supported by the chemical shift of the chelated hydroxyl group (δ 12.70), which is shifted downfield relative to that of the aglycone, eriodictyol (**3**) (Fukai and Nomura, 1990; Horowitz and Jurd, 1961), indicating C-6 substitution, as well as the virtual absence of a batochromic $AlCl_3$ induced shift in the UV spectrum (λ^{MeOH} nm: 289, $+AlCl_3$: 295, Δ 6 nm) compared to that of the aglycone (Sherif et al., 1980) (λ^{EtOH} nm: 289, $+AlCl_3$: 310, Δ 21 nm) (Horowitz and Jurd, 1961).

Diazomethane methylation of the diastereomeric mixture of (*S*)- and (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside (**4**) resulted in an intractable mixture. Methylation with MeI and K_2CO_3, however, resulted in the

[3] HPLC chromatography of the acetylated mixture of **4** on a Chiralcel OD column (25 × 0.46 cm i.d.) with hexane–EtOH–HOAc (30:70:0.12, v/v) as mobile phase and UV detection at 290 nm did afford a separation with peaks at retention times 7.355 and 7.842 min. Postcolumn decomposition occured due the low pH of the mobile phase and no elution could be obtained in the absence of acid.

[4] Owing to the inability to distinguish between the (*R*)- and (*S*)-diastereomers, the resonances of H-3 are indicated as *cis* and *trans*, respectively, instead of *axial* and *equatorial*.

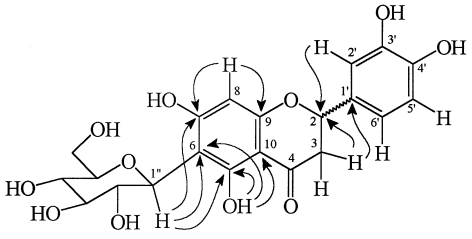


Fig. 1. HMBC correlations.

formation of 2′,3,4,4′,6′-pentamethoxy-3′-β-D-glucopyranosylchalcone (**7**). Chalcone formation from a flavanone in the presence of the mild base K_2CO_3, is analogous to the rapid formation (5–10 min) of chalcones from 4′-hydroxy-7-alkoxy- or 4′-hydroxy-7-*O*-glucosylflavanones in diluted alkali due to the increased acidity of the α-hydrogen relative to compounds with a 7-hydroxyl group (Horowitz and Jurd, 1961). Hydrogen bonding of the 7-OH in **4** and the heterocyclic oxygen of the sugar moiety presumably reduces the release of electron density from the hydroxyl group to the C-4 carbonyl hence ensuring sufficient acidity of the α-hydrogen to facilitate ring opening. The 2′,3,4,4′,6′-pentamethoxy-3′-β-D-glucopyranosylchalcone (**7**), presumably originating from the hitherto unknown free phenolic chalcone (**6**) and fully characterized as the pentamethyl ether acetate (**8**), is a new entry to the known pool of chalcones. The ^{1}H NMR spectrum of **7** (Table 3) revealed the presence of a typical ABX system for the A-ring protons (δ 7.27, *d*, $J = 2.0$ Hz, H-2; δ 6.96, *d*, $J = 8.5$ Hz, H-5 and δ 7.16, *br.dd*, $J = 2.0$ and 8.5 Hz, H-6), a B-ring singlet (δ 6.56, H-5′), five methoxy resonances and the typical α- and β-proton resonances (δ 6.86 and 7.27, both *d*, $J = 16.0$ Hz) of a chalcone. Acetylation yielded 2′,3,4,4′,6′-pentamethoxy-3′-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)chalcone (**8**) as a yellow amorphous solid. Individual sugar resonances (except H-5″) were clearly assignable in the ^{1}H NMR spectrum (Table 3). Duplication of signals due to rotational isomerism [proved by the inversion transfer between the anomeric protons (Coetzee et al., 1995), H-1″ (δ 4.99) exchanged inversely with H-1″ (δ 4.81, 49%) in an n.O.e correlation, whereas H-1″ (δ 4.81) exchanged similarly with H-1″ (δ 4.99, 51%)] explained the presence of eight acetoxy signals and 10 methoxy signals in the ^{1}H NMR spectrum. The FAB mass fragments confirm the proposed structure.

Table 2
^{13}C NMR data (ppm) of diastereomeric mixture **4** at 296 K

C	$(CD_3)_2CO$
2	79.4038
3	43.1536, 42.9208[a]
4	197.052
5	162.644[b]
6	105.283
7	166.031
8	96.2283, 96.0995[a]
9	163.017[a], 162.960[a,b]
10	102.469
1′	130.935, 130.865[a]
2′	114.253, 114.214[a]
3′	145.623
4′	146.000
5′	115.551
6′	118.664
1″	74.8007
2″	81.4111[c]
3″	79.0619[c]
4″	72.9932[c]
5″	70.3912[c]
6″	61.4028

[a] Duplicated resonances.
[b] Allocations interchangeable.
[c] Allocations interchangeable.

2.2. Flavanone formation during the oxidative cyclization of aspalathin (*1*)

Koeppen and Roux (1965) reported that apalathin (**1**) is photochemically converted in both aqueous HOAc and EtOH solutions in ordinary sunlight and in the presence of *oxygen* into both eriodictyol-6-*C*-β-D-glucopyranoside (**4**) and eriodictyol-8-*C*-β-D-glucopyranoside (**5**), the latter apparently due to a Wessely–Moser rearrangement of **4**. No indications as to yield and conversion were given.

Irradiation of aspalathin (**1**) with ultraviolet light at 254 nm for nine days [see also relevant experiments by Kodama et al. (1992)] in D_2O (to permit NMR analysis at regular intervals) gave **4** in a 6% yield. Deuterated analogues (**9** and **10**) were obtained in a 94% yield. The formation of **9** and **10** can be attributed to α-hydrogen/deuterium exchange via enolisation.

The mechanistic sequence depicted in Scheme 1 is proposed as a rationale for the photochemical formation of flavanones **4a** and **4b** from aspalathin (**1**). The triplet state (**12**) presumably permits hydrogen abstraction from ground state aspalathin (**1**) to give a phenoxyl radical (**13**) and subsequently a quinone methide (**16**) (Bradley and Min, 1992; Howard, 1973) via *o*-quinone–quinone methide tautomerism. The electrophilic exo-cyclic carbon of **16** is then trapped in an exo-trig cyclization via a phenolic oxygen of the phloroglucinol ring. Nucleophilic addition to the quinone methide (**16**) resulting from *o*-quinone–quinone methide tautomerism finds a precedent in the work of Cilliers and Singleton (1991). The excellent hydrogen atom donating ability of aspalathin (**1**) (von Gadow et al., 1997) supports the proposed mechanism.

To investigate the effect of the heat component of sunlight, a solution of aspalathin (**1**) in EtOH–H_2O (1:1, v/v) was exposed to oxygen in the dark at room temperature as well as at 30°C. At room temperature no conversion took place, whereas at 30°C a diastereomeric mixture of flavanones **4a** and **4b** was formed in 16% yield and 99% conversion after 6 days. Since oxygen is a prerequisite for oxidative cyclization of aspalathin a similar mechanism as depicted in Scheme 1 is proposed. Formation of the reactive *o*-quinone in-

termediate, **15**, is preceded by phenoxyl radical (**13**) formation via electron transfer from a phenolate moiety to $^3O_2$ and removal of the hydrogen of 3-OH by a superoxide anion.

This biomimetic study of the fermentation process of rooibos thus revealed that the dihydrochalcone, aspalathin (**1**) is converted oxidatively in the presence of light and heat into the flavanones (*S*)- and (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside (**4a** and **4b**). These may further rearrange and/or be oxidized to the corresponding flavones.

The assumption that sunlight is necessary for the processing of rooibos thus seems to be only partly true, as heat energy without the presence of ultraviolet light supports the same process. This implies that quality rooibos tea can be processed under controlled conditions at elevated temperatures and is demonstrated by a pilot study of Joubert and de Villiers (1997).

The demonstration of the oxidative cyclization of the dihydrochalcone aspalathin (**1**) to the corresponding flavanones (**4**) represents a fundamental contribution towards the hitherto neglected field of dihydrochalcone chemistry. Such a chemical transformation of dihydrochalcones to flavanones is unique since flavanones are considered to be equilibrium products of chalcone cyclization under the influence of a chalcone isomerase. The isolation and synthesis of (*S*)- and (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside (**4**) extend the rare series of naturally occurring flavanone C-glycosides (Budzianowski and Skrzypczakowa, 1978; Garg et al., 1978; Bohm, 1994; Hsu and Chen, 1993).

3. Experimental

3.1. General

^1H and ^{13}C NMR spectra were recorded on a Bruker AM-300 spectrometer in acetone-d_6, ethanol-d_6, D_2O or $CDCl_3$ with TMS as internal standard. Mass spectra were recorded on a Kratos MS-80 instrument at 80 eV. TLC was performed on precoated Merck plastic sheets (silica gel 60 F_{254}, 0.25 mm) and the plates sprayed with H_2SO_4–HCHO (40:1, v/v) after development. Preparative plates (PLC), 20 × 20 cm, Kieselgel PF_{254} (1.0 mm) were air-dried and used without prior activation. Compounds were recovered from absorbent with 50% (v/v) Me_2CO in MeOH. CC was on Sephadex LH-20, silica (Flash CC), or cellulose. Methylations were performed with an excess of CH_2N_2 in MeOH–Et_2O at −15°C for 48 h, while acetylations were in Ac_2O–pyridine at ambient temperatures. Evaporation were done under reduced pressure at ambient temperatures in a rotary evaporator and freeze-drying of aqueous solutions on a Virtis 12 SL freezemobile. HPLC separations were performed on a Hewlett-Packard HPLC system comprising of a G1310 Iso Pump, G1313A ALS autosampler and a G1314A UV/Vis detector. Data were recorded and integrated with a Hewlett-Packard model 35900E A/D converter interfaced to a computer workstation running Hewlett-Packard LC ChemStation (2D) version A.04.02 software.

3.2. Isolation

A portion (270 mg) of fraction C10 from MPLC

Table 3
^1H NMR data of **7** and **8** at 300 MHz and 296 K[a]

Ring	H	**7** (Acetone-d_6)	**8** ($CDCl_3$)
A	2	7.27 (*d*, 2.0)	7.07 (*d*, 2.0)
	5	6.96 (*d*, 8.5)	6.82 (*d*, 8.5)
	6	7.16 (*br.dd*, 8.5, 2.0)	7.03 (*dd*, 8.5, 2.0)
B	5	6.56 (*br.s*)	6.29, 6.23[b] (s)
Glc	1	4.77, 4.54[b] (*br.d*, 10.5)	4.99, 4.81[b] (*d*, 10.5)
	2	4.36–4.10 (*m*)	6.03, 5.84[b] (*dd*, 9.5, 9.5)
	3	4.36–4.10 (*m*)	5.28, 5.26[b] (*dd*, 9.5, 9.5)
	4	4.36–4.10 (*m*)	5.20, 5.17[b] (*dd*, 11.5, 9.5)
	5	4.36–4.10 (*m*)	ND[c]
	6	3.63 (*dd*, 12.0, 5.5)	4.31 (*dd*, 12.5, 4.5), 4.24[b] (*dd*, 12.5, 5.0), 4.12 (*dd*, 12.5, 2.5), 4.06[b] (*dd*, 12.5, 2.5)
	α	6.86 (*d*, 16.0)	6.86, 6.83[b] (*d*, 16.5)
	ß	7.27 (*d*, 16.0)	7.33 (*d*, 16.5)
	–OMe	3.87, 3.83, 3.82, 3.78, 3.69 (each *s*)	3.99 (×2), 3.93, 3.88, 3.87, 3.85, 3.77, 3.76, 3.74, 3.72[b] (each S)
	–OAc	–	2.04 (×2), 2.01 (×2), 1.98, 1.97, 1.81, 1.79[b] (each *s*)

[a] Splitting patterns and *J*-values are given in parentheses.
[b] Duplicated resonances due to rotational isomerism.
[c] ND: not distinguishable.

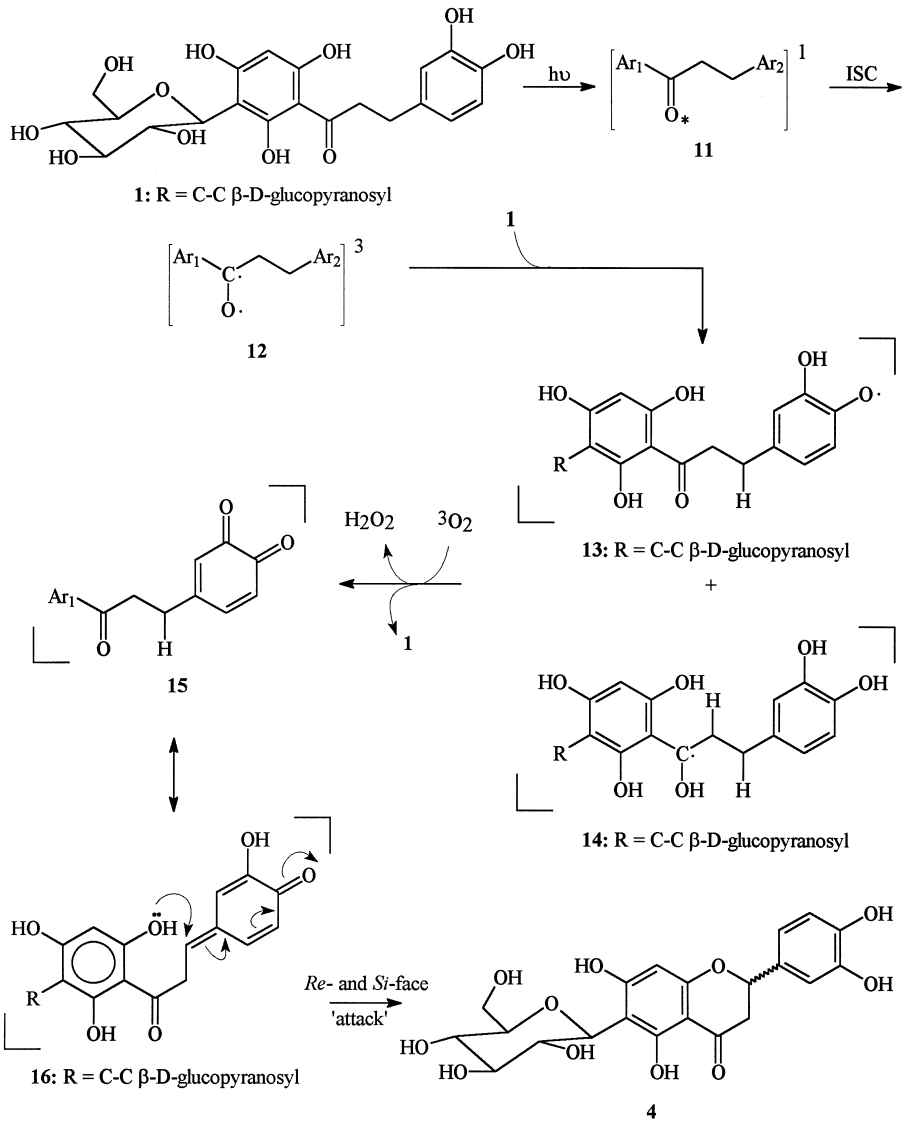
separation of the acetone extract (Rabe et al., 1994) was subjected to CC on cellulose (MN 300, 2 × 40 cm column, 13 ml fractions) in H_2O to give a subfraction which contained compound **4** (84 mg).

3.3. (S)- and (R)-Eriodictyol-6-C-β-D-glucopyranoside (**4a** and **4b**)

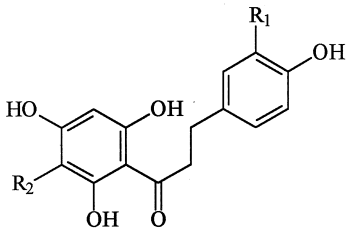
Yellow amorphous solid. Found [M^+] 450.1161 $C_{21}H_{22}O_{11}$ requires 450.1162. UV λ_{max} (MeOH) nm: 289, 335; λ_{max} (MeOH + $AlCl_3$) nm: 295 sh. CD (MeOH, *c* 0.0324): $[\theta]_{330}$ +0.5, $[\theta]_{312}$ +0.29, $[\theta]_{301}$ +0.17, $[\theta]_{297}$ +0.22, $[\theta]_{291}$ +0.079, $[\theta]_{285}$ +0.33, $[\theta]_{271}$ +0.11, $[\theta]_{262}$ −0.0057. ^{1}H NMR: Table 1. ^{13}C NMR: Table 2. FAB MS, *m/z* (rel. int.): 450 [M^+] (16), 288 (8), 286 (2), 136 (78).

3.4. 2′,3,4,4′,6′-Pentamethoxy-3′-β-D-glucopyranosylchalcone (7)

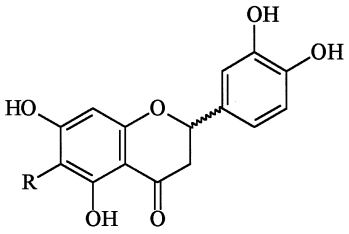
The flavanone glycoside (**4**, 50 mg) was methylated with MeI (80 equiv.) in dry Me_2CO/K_2CO_3 (40 equiv.) under N_2 and the mixture refluxed for 15 h. Filtration and concentration in vacuo, followed by PLC [hexane–C_6H_6–Me_2CO–MeOH (1:3:5:1)] afforded the title compound as a yellow amorphous solid (**7**, 8.3 mg, 14.5%). ^{1}H NMR: Table 3. Acetylation of **7** (8.3 mg) afforded the pentamethyl ether acetate (**8**) as a yellow amorphous solid (**8**, 8.3 mg, 27 %). Found [M^+] 688.2432 $C_{34}H_{40}O_{15}$ requires 688.2418. ^{1}H NMR: Table 3. FAB MS, *m/z* (rel. int.): 660 (11), 525 (100), 495 (26), 483 (9), 453 (37), 423 (12), 381 (18), 331 (15), 289 (23), 136 (80).



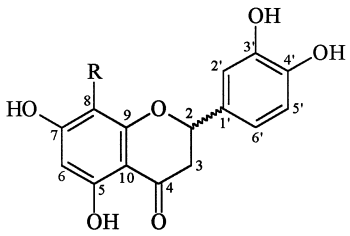
Scheme 1. Proposed mechanism for the photochemical formation of **4**.



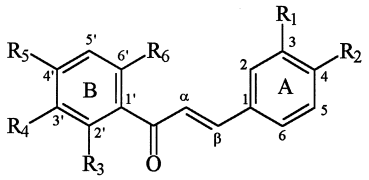
1: R_1 = OH; R_2 = C-C β-D-glucopyranosyl
2: R_1 = H; R_2 = C-C β-D-glucopyranosyl



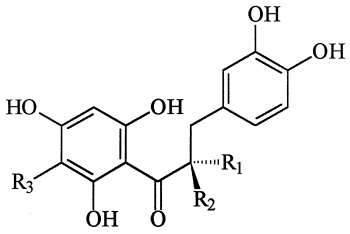
3: R = H
4: R = C-C β-D-glucopyranosyl
4a: ▬ = ▬ ; R = C-C β-D-glucopyranosyl
4b: ▬ = ┈┈ ; R = C-C β-D-glucopyranosyl



5: R = C-C β-D-glucopyranosyl



6: R_1 = R_2 = R_3 = R_5 = R_6 = OH;
R_4 = C-C β-D-glucopyranosyl
7: R_1 = R_2 = R_3 = R_5 = R_6 = OMe;
R_4 = C-C β-D-glucopyranosyl
8: R_1 = R_2 = R_3 = R_5 = R_6 = OMe;
R_4 = 2,3,4,6-tetra-*O*-acetyl- β-D-glucopyranosyl



9: R_1 = H; R_2 = D; R_3 = C-C β-D-glucopyranosyl
10: R_1 = D; R_2 = H; R_3 = C-C β-D-glucopyranosyl

3.5. Oxidative cyclization of aspalathin by irradiation

Irradiation of aspalathin (**1**, 40 mg), dissolved in D_2O (3 ml, for ^1H NMR monitoring) saturated with O_2, for 9 days at 254 nm in a sealed tube, afforded the diastereomeric flavanones (**4**, 6%), the ^1H NMR data of which were in agreement with those of the natural products, and the deuterated (α- and *β*-positions) aspalathin (94%).

3.6. Oxidative cyclization of aspalathin induced by heat

Aspalathin (**1**, 11 mg) was dissolved in 50% EtOH–H_2O (5 ml). The mixture was saturated with O_2 on a regular basis and stirred at 30°C for 6 days. NMR-analysis revealed the presence of the diastereomeric flavanones (**4**, 16%), of which the chemical shifts and coupling constants were identical to those of the natural products (vide supra), together with unreacted aspalathin (**1**, 9.24 mg). Control experiments were performed under argon and at low temperatures.

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